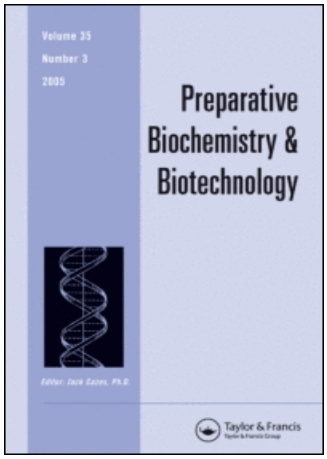

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Isolation and Characterization of the P5 Adhesin Protein of **Haemophilus parasuis** Serotype 5

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Abstract: *Haemophilus parasuis* is a Gram-negative respiratory pathogen of young pigs that colonizes the upper respiratory tract and produces a number of symptoms collectiviely described as Glässer's disease. Recently, an *H. parasuis* P5-like outer membrane adhesin protein homologous to *H. influenzae* P5 was identified. The P5 adhesin was partially purified by anion exchange and size-exclusion chromatography. Final purification for functional studies was performed by elution of the protein from a polyacrylamide gel. Identification of the protein as a P5 adhesin homolog of *H. influenzae* was confirmed by N-terminal sequencing. The P5 protein had a molecular mass of 32,000 and a pI of 5.5. Unlike the *H. influenzae* P5 adhesin, the *H. parasuis* P5 protein did not bind carcinoembryonic antigen.

Keywords: Haemophilus parasuis, P5 outer membrane protein, Adhesin

INTRODUCTION

A number of species from the genus *Haemophilus* are known to colonize the respiratory tracts of their hosts. *H. parasuis*, the causative agent of Glässer's disease, is one of these species.[1,2] Although little is known about the initial colonization events, reports show that *H. parasuis* caused changes of the

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nasal mucosa of pigs in a similar fashion as *H. influenzae* produced in human nasal mucosa.[3,4] Colonization factors have been identified for *H. influenzae* and among these are the surface-exposed outer membrane proteins P1, P2, P4, P5, and P6.[5] The role of outer membrane proteins in colonization and virulence is of particular interest to this study. The P5 protein is one of the major outer membrane proteins of *H. influenzae*.[6,7] Surface exposed regions of the P5 protein of *H. influenzae* were variable from one strain to another and this variability is proposed to help the organism evade the immune response.[8–10] A recent report indicated that region 4 of P5 contained a highly immunodominant but non-protective epitope which dampened the immune response to a subdominant but protective epitope in region 3.[11] In addition, P5 has been shown to bind a member of the carcinoembryonic antigen family, CEACAM1 (CD66), and P5-expressing strains are capable of efficient adherence to CHO cells expressing CEACAM1, whereas P5-deficient strains demonstrated minimal adherence.[9] Furthermore, a similar protein expressed by *Neisseria gonorrhoeae* was responsible for immunosuppression when bound to CEACAM1.[12] These authors speculated that immunosuppression may therefore also occur in *H. influenzae*.[12]

Given the similarities that exist between members of the genus *Haemophilus*, the intent here was to characterize the P5 protein in *H. parasuis*. Specifically, the objectives of this study were to purify the P5 protein using traditional chromatographic techniques and to characterize the P5-like protein by N-terminal sequencing, isoelectric focusing (IEF), and matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. In addition, we sought to determine the interaction between the P5 protein and carcinoembryonic antigen (CEA) by developing an immunoblot method.

EXPERIMENTAL

Bacterial Strains

H. parasuis was a field isolate (serotype 5, strain IA84-297755) from the lung of an infected piglet kindly provided by Vicki Rapp-Gabrielson (formerly of Schering-Plough, Omaha, NE). *H.* parasuis reference strains 2 and 3 have been described previously.[13] Strains were grown on Casman agar, supplemented with 5% horse serum (Gibco-BRL, Carlsbad, CA), and overlaid with 0.016% NAD (Sigma Chemical Co., St. Louis, MO.) using 5% CO_2 at 37°C. Colonies were selected and grown in a liquid media consisting of Freys medium (Sigma Chemical Co., St. Louis, MO.) supplemented with 20% horse serum (Gibco-BRL, Carlsbad, Ca) and 0.016% NAD (Sigma Chemical Co., St. Louis, MO.). Strains were stored in this media with 10% glycerol at −80°C. *H. influenzae* strains d1 (P5 positive) and d2 (P5 negative), which are isolates from sputum samples of a single patient with chronic bronchitis, were also used.[7] The *H. influenzae* strains were

cultured in BHI with 10 μg/ml haemin (x-factor) and 10 μg/ml NAD. Outer membrane protein fractions were prepared as described earlier.[14]

Anion Exchange and Size Exclusion Chromatography

The *H. parasuis* serotype 5 field isolate (strain IA84-29755) was sonicated for 5 minutes on ice, using a Branson sonicator (Branson Ultrasonics Corp., Danbury, CT) equipped with a microtip. The supernatant was recovered after centrifugation at 4,000 x g for 20 minutes, adjusted to a concentration of 1 mg/mL protein and dialyzed against 12.5 mM N-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid (TAPS), 10 mM NaCl, 0.05% 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate (CHAPS), pH 8.5. The dialyzed supernatant (10 mg) was applied to a quaternary methyl amine (QMA) anion exchange column (2.6 × 20 cm) (Millipore, Billerica, MA). Fractions were collected and monitored by absorbance at 280 nm. Bound proteins were eluted with a gradient elution to 12.5 mM TAPS, 400 mM NaCl, 0.05% CHAPS, pH 8.5. Fractions were analyzed by immunoblotting (see below) with the anti-P5 monoclonal antibody and pooled based on reactivity. The pooled fractions were then applied to a Sephacryl-100 HR column (2.6 × 50 cm) (Pharmacia, Uppsala, Sweden). Again, fractions were collected and monitored by absorbance at 280 nm, as well as by immunoblotting with the anti-P5 monoclonal antibody.

Extraction of P5 from Polyacrylamide Gel

After purification by the QMA and Sephacryl-100 HR matrices, fractions were pooled and applied to a 4-12% SDS-PAGE gel (InVitrogen, Carlsbad, CA). The 32 kDa P5 protein band was cut from the gel and minced into smaller pieces. The minced pieces were placed in a Nanosep 100 kDa centrifugal device (Pall Life Sciences, Ann Arbor, MI) and 0.5 ml of elution buffer (125 mM Tris with 0.5% SDS, pH 6.9) was added. The device was centrifuged at 8,000×g for 20 minutes. The filtrate was recovered and a second elution was performed. Again, the filtrate was collected and added to the filtrate from the first elution. The pooled filtrate was concentrated using a 3 kDa centrifugal concentrator (Millipore, Billerica, MA) and analyzed for purity by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

Protein Determination

The protein concentrations were determined using a bicinchoninic acid reagent kit (Pierce, Rockford, IL) according to the manufacturer's protocol. Bovine serum albumin (Pierce, Rockford, IL) was used as the standard.

SDS-PAGE and Immunoblotting

Samples were boiled for 5 minutes in a SDS treatment buffer consisting of 62.5 mM Tris, 10% glycerol, 5% 2-mercaptoethanol, 1% SDS, and 0.001% bromophenol blue. Samples (7.5 μg/protein well) were applied to 4–12% gradient SDS-polyacrylamide gels (InVitrogen, Carlsbad, CA). Gels were stained with Coomassie Brilliant Blue R250 or transferred electrophoretically to 0.45-μm polyvinyledene difluoride (PVDF) (Millipore, Billerica, MA) for immunoblotting. Immunoblots were blocked with 0.25% fish gelatin in wash buffer consisting of 1.5 mM KH_2PO_4, 20 mM Na_2HPO_4, 125 mM NaCl, 3 mM KCl, 0.05% Tween-20 (pH 7.2). For the blots aimed at detecting P5, the blocked membranes were incubated with 1:5,000 dilutions of anti-P5 monoclonal antibody (4BF8), and 1:5,000 dilutions of rabbit anti-mouse IgG horseradish peroxidase-labeled (Jackson ImmunoResearch, West Grove, PA) sequentially. The membranes were washed between steps by rotating with 0.05% Tween 20 in PBS. Blots were developed with 3,3′,5,5′-tetramethylbenzidine (TMB) (Kirkegaarde and Perry Labs, Gaithersburg, MD) as the substrate.

Protein Sequencing

Following SDS-PAGE, gels were blotted electrophoretically to PVDF (Millipore, Billerica, MA) and stained with Coomassie Brilliant Blue R250. Bands were excised from the PVDF and the N-terminal amino acid sequence was determined by automated Edman sequencing with a model 494 Procise protein/peptide sequencer (Applied Biosystems, Foster City, CA) at the Iowa State University Protein Facility. Sequence homologies were determined by using the BLAST server of the National Center for Biotechnology Information.[15]

Carcinoembryonic Antigen (CEA) Immunoblot

SDS-PAGE gels were transferred electrophoretically to 0.45-μm PVDF (Millipore, Billerica, MA) for immunoblotting. Immunoblots were blocked with 0.25% fish gelatin in wash buffer consisting of 1.5 mM KH_2PO_4, 20 mM Na_2HPO_4, 125 mM NaCl, 3 mM KCl, 0.05% Tween-20 (pH 7.2). The blocked membranes were incubated with 0.5 μg/mL CEA (BioDesign, Saco, MD) diluted in block buffer. The membranes were washed by rotating with 0.05% Tween 20 in PBS. After washing, the membranes were incubated with a polyclonal goat anti-CEA (BioDesign, Saco, MD) diluted 1:25,000 in block buffer. After washing again, the membranes were incubated with a horse-radish peroxidase labeled polyclonal mouse anti-goat IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:10,000

in block buffer. After another washing step, the blots were developed with 3,3′,5,5′-tetramethylbenzidine (TMB) (Kirkegaarde and Perry Labs, Gaithersburg, MD) as the substrate.

Isoelectric Focusing

The purified P5 protein (7.5 μg) was subjected to isoelectric focusing using a Novex IEF system, pH 3–10 (InVitrogen, Carlsbad, CA). The gel was run for one hour at 100 V, one hour at 200 V, and finally 500 V for 30 minutes. Fixing was accomplished with sulphosalicylic acid and TCA for 30 minutes followed by Coomassie R-250 staining. The gel was destained until clarity was achieved.

Mass Spectrometry

A protein-containing gel plug was removed with a blunt-cut 20 gauge needle, digested with trypsin using an automated digester (ProGest, Genomics Solutions, Ann Arbor, MI) according to the manufacturer's instructions. Recovered peptide fragments were eluted from a C_{18} ZipTip (Millipore, Billerica, Mi) using a saturated solution of 4-hydroxycinnamic acid in 30% acetonitrile, 0.1% trifluoroacetic acid, and analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer (Voyager DE Pro, Applied Biosystems, Foster City, CA) of the Iowa State University Protein Facility. Monoisotopic masses were used to search the NCBI non-redundant database using Prospector MS FIT search algorithm available at www.ucsf.edu.

Monoclonal Antibody

The cross reactive monoclonal antibody (4BF8) used here has been described earlier.[8] It has been shown to react with several variants of P5 suggesting that it does not react with the variable surface exposed loops.

RESULTS

Purification of P5

Using the QMA anion exchange method with an equilibration buffer of 12.5 mM TAPS, 10 mM NaCl, 0.05% CHAPS, pH 8.5 resulted in the fractionation of the higher pI proteins which included the P5 protein. The optimum resolution was achieved with a load rate of 2.5 mg of total protein per ml of

QMA resin. At this total protein load, a P2-like protein was observed in the unbound fraction while the P5-like protein bound to the QMA matrix and was eluted with a gradient of NaCl equivalent to 150 mM (Fig. 1). An additional purification step utilizing size exclusion chromatography was employed. However, the use of the Sephacryl 100-HR matrix (Fig. 2) did not result in the isolation of pure P5. Instead, the P5 protein was repeatedly co-purified with a 61 kDa protein (Fig. 3, lane 3) identified by N-terminal sequencing as an immunoglobulin heavy chain variable region. Isolation of the pure P5 protein was only achieved by excising the 32 kDa band from an SDS-PAGE gel and extracting it using ultrafiltration techniques (Figure 3, lane 4 and Fig. 4, lane 2).

Identification of P5 by N-Terminal Sequencing and Peptide Mass Fingerprinting

The N-terminal sequence APQANSFYVG of the 32 kDa P5-like protein of *H. parasuis* serotype 5 was homologous to the *H. influenzae* 32 kDa P5 N-terminal sequence of APQENTFYAGV.[16] The *H. parasuis* sequence contained conservative substitutions of A_4, S_6 and V_9 for residues E_4, T_6 and A_9 of the *H. influenzae* sequence.

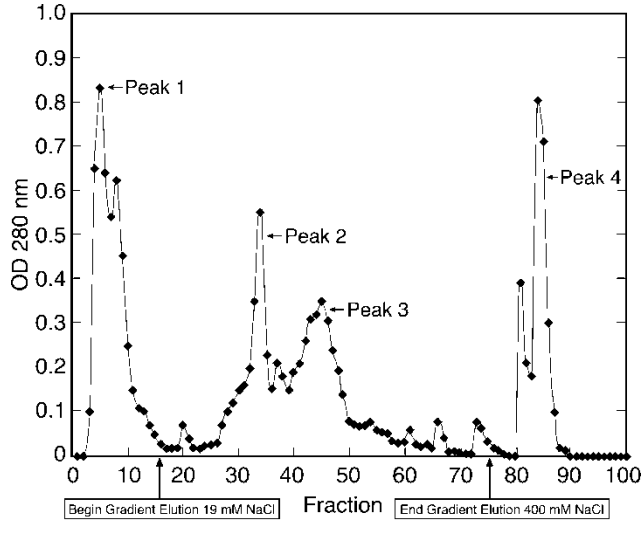

Figure 1. Anion exchange chromatography of *H. parasuis* serotype 5 whole cell sonicate. Protein supernatant was loaded onto the QMA anion exchange column equilibrated with 25 mM TAPS, 10 mM NaCl, 0.05% CHAPS, pH 8.5. Bound proteins were eluted with a gradient elution to 12.5 mM TAPS, 400 mM NaCl, 0.05% CHAPS, pH 8.5. The P2 protein was found in peak 1, whereas the P5 protein eluted in peak 3. The run was monitored at 280 nm.

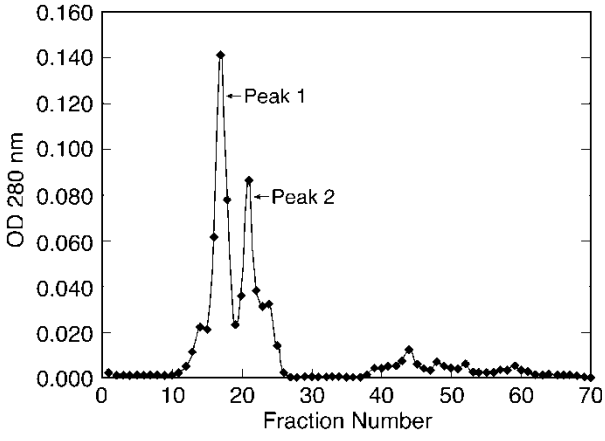

Figure 2. Size-exclusion chromatography. Pooled fractions from the anion exchange chromatography run were loaded onto a Sephacryl 100-HR column, equilibrated with 0.2 M Tris, 0.3 M NaCl, 0.1% sodium azide, pH 7.4. The P5 protein was found in peak 2. The run was monitored at 280 nm.

Interestingly, homology searches using the peptide mass data revealed homologies with four *H. influenzae* P2 protein variants. The top scoring (MOWSE score of 68.5) homolog in the five top matches showed a molecular weight of 40,098, pI of 8.4 and 67% mass coverage (Accession No. 2981123).

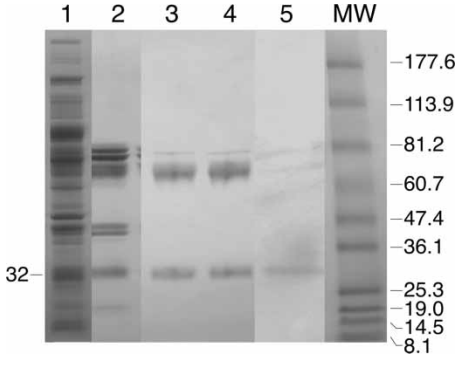

Figure 3. SDS-PAGE of the various P5 protein fractions after the purification steps. Lane 1, whole cell sonicate supernatant of *H. parasuis* serotype 5; lane 2, peak 2 fraction after anion exchange chromatography; lanes 3 and 4, peak 2 fraction after Sephacryl-100 HR chromatography; lane 5, after PAGE gel extraction; 6, molecular weight standard. Samples were separated with a 4-12% SDS-PAGE gel and stained with Coomassie R-250 as described in the text. Molecular weights are indicated in kilo-Daltons.

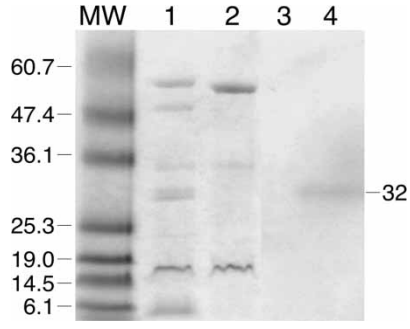

Figure 4. SDS-PAGE *H. influenzae* outer membrane proteins and *H. parasuis* P5 protein. Lanes: 1, molecular weight standard; 2, *H. influenzae* strain d1 (P5 positive); 3, *H. influenzae* strain d2 (P5 negative); Gel-purified P5 protein from *H. parasuis* serotype 5. Samples were separated with a 4–12% SDS-PAGE gel and stained with Coomassie R-250 as described in the text. Molecular weights are indicated in kilo-Daltons.

Physical Characterization of Purified P5

SDS-PAGE analysis of the purified P5-like protein showed a single band by Coomassie blue staining (Fig. 3, lane 4 and Fig. 4, lane 3). The molecular weight of the band was 32 kDa and was identified as P5 by N-terminal sequencing. Isoelectric focusing produced a pI of 5.5 for the native protein (Fig. 5). Immunoblotting with the protein and the monoclonal anti-P5 antibody also resulted in the identification of a single band of 32 kDa (Fig. 6).

CEA Immunoblot

The purified P5 protein from *H. parasuis* and the outer membrane proteins from *H. influenzae* strains d1 and d4 were assayed with the CEA immunoblot method in order to assess its ability to bind with the CEA protein (Fig. 7). CEA

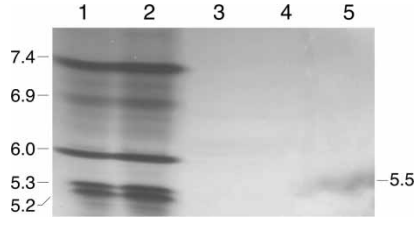

Figure 5. Isoelectric focusing gel of the gel-purified P5 protein. Lane: 1, IEF standards; lane 2, gel-purified P5 protein from *H. parasuis* serotype 5. Numbers in the margin are pI values for the standard proteins.

binding was observed with *H. influenzae* strain d1 with both the 55 kDa P2 protein and with the 48 kDa P5 protein, whereas the d4 strain which lacks the P5 protein showed binding only with the 55 kDA P2 protein. Interestingly, unlike the results obtained with *H. influenzae*, the 32 kDa P5-like protein from *H. parasuis* did not bind the CEA antigen.

DISCUSSION

This report describes the purification and characterization of a P5-like protein of *H. parasuis* that is homologous to the *H. influenzae* P5 protein by N-terminal sequencing and monoclonal antibody reactivity. We also used an immunoblot method to determine if CEA binds the P5 outer membrane protein as was reported for *H. influenzae*.[12]

In a previous study,[16] an *H. influenzae* P5 protein homolog was identified in *H. parasuis.* One of the aims of this study was to purify the P5 protein from the outer membrane of a virulent *H. parasuis* field isolate. By using the theoretical pI of the *H. influenzae* P5 protein from the published sequence (*H. influenzae* Accession number NP438308) and the Protein Tools of Expasy Molecular Biology Server, a purification strategy was designed. The pI for the P5 protein was 9.5. A second outer membrane protein of *H. influenzae* P2 (Accession No. U32796) showed a theoretical pI of 9.0. A purification strategy for the separation of these two proteins involved anion exchange chromatography using an equilibration buffer with a pH near the pI of the P2 protein. While the P2 and P5 proteins could be

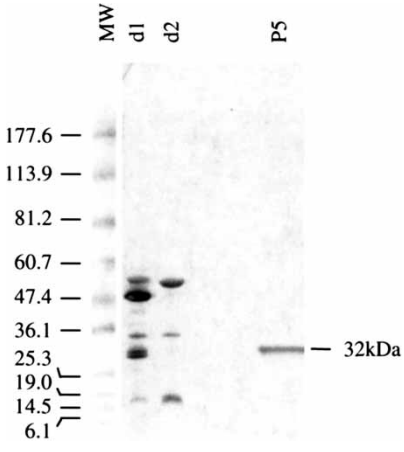

Figure 6. Anti-P5 (4BF8) immunoblot of *H. influenzae* strains d1 and d2 and the P5 protein after extraction. Samples were separated with a 4–12% SDS-PAGE gel, blotted to PVDF, and probed with anti-P5 monoclonal antibody (4BF8) as described in the text. Molecular weights are indicated in kilo-Daltons.

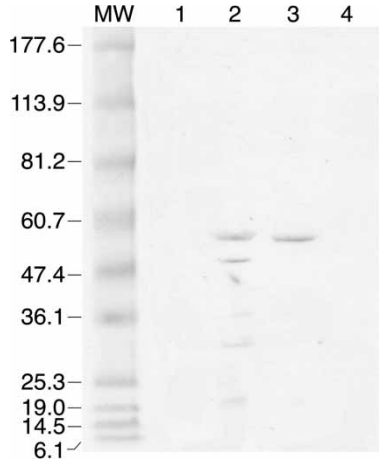

Figure 7. CEA immunoblot of *H. influenzae* strains d1 and d2 and the purified P5 protein. Proteins were separated with a 4–12% SDS-PAGE gel, blotted to PVDF, and probed with human CEA, goat anti-CEA antibody, and peroxidase labeled mouse anti-goat IgG as described in the text. Lane 1, no protein loaded; lanes 2 and 3, outer membrane proteins from *H. influenzae* strains d1 and d2, respectively; lane 4, purified *H. parasuis* P5 protein. Molecular weights are indicated in kilo-Daltons.

separated with the anion exchange method (Fig. 1), a number of other proteins were co-purified with the P5 protein (Fig. 3, lane 2). Further purification was accomplished by size exclusion chromatography. However, the P5 protein co-eluted with a protein of approximately 61 kDa. In order to obtain a pure P5 protein, it was separated electrophoretically and excised, then eluted from the gel. The purified P5 protein was analyzed by SDS-PAGE for purity and a single band was found at 32 kDa. The band was identified as the P5 homolog of *H. influenzae* by N-terminal sequence analysis and database searching. However, analysis of the 32 kDa band by peptide mass finger printing revealed that the 32 kDa protein was partially homologous to *H. influenzae* P2. These data suggest that some similarity exists between the P2 and P5 proteins of *H. parasuis* and *H. influenzae* but that N-terminal sequence preferentially identified the P5 homolog. We are currently performing further sequence studies on the *H. parasuis* P5 protein by tandem mass spectrometry. The molecular weight of the *H. parasuis* P5 protein is comparable to the molecular weight of the *H. influenzae* P5 protein which ranges from 27 to 35 kDa.[5] Isoelectric focusing produced a pI of 5.5 for the native *H. parasuis* protein. This is much lower than the predicted pI of 9.5 by using the Protein Tools of the Expasy Molecular Biology Server for the *H. influenzae* P5 protein. Elution of the *H. parasuis* P5 protein required 150 mM of NaCl, which is consistent with a protein of low pI. Although the P5 proteins of *H. parasuis* and *H. influenzae* are from different species, they

are both recognized by the anti-*H. influenzae* P5 monoclonal antibody, suggesting the target epitope is highly conserved.

Because the *H. influenzae* P5 protein is known to bind CEA,[9,17] an immunoblot method was developed here to assess the ability of the outer membrane proteins to bind CEA. The outer membrane preparations from *H. influenzae* served as controls for this assay and reactivity was observed to the P5 and P2 outer membrane proteins. However, no binding was observed with the purified *H. parasuis* P5 protein or with the P5 protein from the type strains, whereas binding to the *H. parasuis* P2 was observed.[16] Perhaps antigenic variability is responsible for the lack of binding observed here. The P5 protein is known to be highly variable in *H. influenzae* and the CEA antigen used here was from a human source. Since *H. influenzae* affects human hosts, perhaps this makes it specific for human CEA. Although CEA from pig lymphocytes is not commercially available, it would be interesting to test CEA from pig lymphocytes in the binding assay with *H. parasuis* P5.

CONCLUSIONS

We have purified and characterized a P5-like protein from *H. parasuis* that is thought to be involved in colonization by *H. parasuis* of the upper respiratory tract of pigs. Unlike the *H. influenzae* P5 protein, the *H. parasuis* P5 protein does not bind the CEA antigen.

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